

## BEST AVAILABLE COPY

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>G01N 33/543 // C12Q 1/68</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 97/00446</b> <b>(43) International Publication Date:</b> 3 January 1997 (03.01.97)
<b>(21) International Application Number:</b> PCT/SE96/00779 <b>(22) International Filing Date:</b> 14 June 1996 (14.06.96) <b>(30) Priority Data:</b> 9502196-0      16 June 1995 (16.06.95)      SE <b>(71)(72) Applicant and Inventor:</b> LANDEGREN, Ulf [SE/SE]; Eksoppsvägen 16, S-756 46 Uppsala (SE). <b>(74) Agents:</b> ALDENBÄCK, Ulla et al.; Dr. Ludwig Brann Patentbyrå AB, P.O. Box 1344, S-751 43 Uppsala (SE).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> IMMUNOASSAY AND KIT WITH TWO REAGENTS THAT ARE CROSS-LINKED IF THEY ADHERE TO AN ANALYTE		
<b>(57) Abstract</b> <p>The present invention relates to an immunological test kit and immunoassay using a first immobilized antibody having affinity for a specific antigen. The invention is characterized by a second and third antibody being specific for different determinants of the antigen and modified with cross-linkable oligonucleotides. For detection, the oligonucleotides are amplified, whereby only such oligonucleotides will be amplified which have been cross-linked to each other. In this way unspecific background is avoided and detection is possible down to single molecules.</p> <div data-bbox="889 1176 1364 1722" data-label="Diagram"> </div>		

B3

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

WO 97/00446

PCT/SE96/00779

**IMMUNOASSAY AND KIT WITH TWO REAGENTS THAT ARE CROSS-LINKED IF THEY  
ADHERE TO AN ANALYTE**

**Technical field**

The present invention relates to ultrasensitive immunoassays. More specifically, it relates to immunological test kits and processes for immunological detection of a specific antigen. In the present invention, the fields of immunology and molecular genetics are combined.

**Background of the invention**

Immunoassays represent powerful tools to identify a very wide range of compounds, such as antigens and antibodies. Examples of immunoassays are ELISA (enzyme linked immunosorbent assay), EIA (enzyme immunoassay), and RIA (radio immunoassay). Common to all these immunoassays, is that detection sensitivity is limited by the affinity of typical antibodies.

With the prior art immunoassays, detection is not possible below a certain number of molecules, because the background, i.e. unspecifically bound material, interferes with the results. Detection of very low numbers of antigen is becoming increasingly important, especially for diagnostic applications. Therefore, further developments in sensitivity as well as specificity of immunological assays are desired.

Cantor et al, Science, Vol. 258, 2 Oct. 1992, have previously reported the attachment of oligonucleotides to antibodies in order to permit detection of such antibodies having bound antigen in immune reactions. A streptavidin-protein A chimera that possesses tight and specific binding affinity for both biotin and immunoglobulin G was used to attach biotinylated DNA specifically to antigen-monoclonal antibody complexes that had been immobilized on microtiter plate wells. Then, a segment of the attached DNA was amplified by PCR (Polymerase Chain Reaction). Analysis of the PCR products by agarose gel electrophoresis after staining with ethidium bromide allowed detection of 580 antigen molecules ( $9,6 \times 10^{-22}$  moles) which is a significant improvement compared

WO 97/00446

PCT/SE96/00779

2

to, for example, conventional ELISA.

However, in Cantor et al., the labeled DNA-antibody complexes are assembled in situ during the assay. This can create variable stoichiometry in the assembly of the components and in the attachment of the DNA label. Moreover, extra steps are required for addition of biotinylated reagents and binding proteins. Numerous wash steps are also needed to remove excess reagents and to free assay components of non-specifically bound reagents.

Hendrickson et al., Nucleic Acids Research, 1995, Vol 23, No.3, report an advancement of the Cantor et al. assay that reduces complexity. This is achieved through labeling antibody with DNA by direct covalent linkage of the DNA to the antibody. In this approach, the analyte specific antibody and the 5' amino modified DNA oligonucleotide are independently activated by means of separate heterobifunctional cross-linking agents. The activated antibody and DNA label are then coupled in a single spontaneous reaction.

International patent publication no. WO 91/17442 describes a molecular probe for use as a signal amplifier in immunoassays for detecting i.a. antigens. The probe comprises an antibody, a double stranded polynucleotide functioning as a promoter for a DNA dependend RNA polymerase, and a single or double stranded template for the promoter. The transcription product is quantified and correlated to the amount of present antigen in a sample.

However, in all three of the above described immunoassays the attached DNA is only used as a marker by being amplified to detectable levels. There is no distinction between oligonucleotides attached to antibodies having bound antigen and oligonucleotides attached to antibodies not having bound antigen, i.e. those being non-specifically trapped. Non-specifically trapped antibodies give rise to an undesired background signal and limits the minimum number of antigen molecules that can be detected and it will not be possible to distinguish between false positive and

WO 97/00446

PCT/SE96/00779

3

true positive results below a certain number of antigen molecules. Commonly, solid supports such as microtiter plates, are used for the reactions. According to prior art, there will always be an excess of oligonucleotide-labeled antibody that cannot be removed from the solid support by adding background-lowering agents and by repeated wash steps.

#### Summary of the invention

The present invention enables detection of extremely low numbers of antigenic molecules, even down to a single molecule. The invention provides reliable immunoassays in situations where insufficient numbers of antigens are available for conventional assays.

According to a first aspect of the invention, there is provided an immunological test kit comprising a first immobilized reagent having affinity for a specific macromolecule, such as a protein. Furthermore, the test kit comprises a second and a third affinity reagent specific for different determinantes of said macromolecule, and modified with crosslinkable compounds enabling a) conjugation of said second and third affinity reagent only when both are bound to the said, same macromolecule, and b) detection by amplification.

According to a preferred embodiment of the invention, the affinity reagents are antibodies and the crosslinkable compounds are oligonucleotide extensions attached to the second and third antibody, respectively. The macromolecule is in this case a specific antigen.

According to a second aspect of the invention there is provided an immunoassay for detection of a specific antigen, comprising the following steps:

- a) contacting a sample suspected of containing said specific antigen with a first antibody linked to a solid support, said first antibody being specific for a first epitope on the antigen,
- b) washing off excess reagents,

WO 97/00446

PCT/SE96/00779

4

- c) incubating with a solution of a second and a third antibody specific for a second and third epitope of said antigen, and modified with crosslinkable oligonucleotides enabling conjugation of said second and third antibody when both are bound to the said, same antigen,
- d) washing off excess reagents,
- e) amplifying said crosslinked oligonucleotides, and
- f) detecting the amplified products.

Products from the amplification reaction only result when two antibodies, i.e. the second and the third, have bound to the same antigen. Thus, amplification is specific for antibodies having bound to antigen. Non-specifically trapped antibodies do not give rise to any signal.

#### Detailed description of the invention

The present invention will be described more detailed below with reference to the accompanying drawings, in which

Fig. 1 is a schematic view of the principles of the immunoassay according to the invention, and

Fig. 2 shows chemical coupling of amino-modified oligonucleotides to macromolecules.

In Fig. 1 there is shown an immobilized antibody to a specific antigen applied together with two other antibodies, specific for other determinants on the same antigen. Besides antibodies other specifically interacting species with a known affinity, such as lectins, receptors, single chain antibodies, cofactors, oligonucleotides and other non-proteins, can be used in the invention.

The interacting species are modified with crosslinkable compounds in the form of an interacting pair, preferably short oligonucleotide extensions. Upon the coordinated binding of several so modified antibodies, oligonucleotides of neighbouring antibodies are conjugated to each other. The conjugation may or

WO 97/00446

PCT/SE96/00779

5

may not necessitate an enzymatic ligation step depending on the orientation of the oligonucleotide extensions.

If the conjugation is between free 3' and 5' ends ligation is necessary, such as by T4 RNA ligase or T4 DNA ligase. To facilitate the conjugation, it is convenient to use a stretch of oligonucleotides base pairing to and, thereby, juxtaposing the free ends of the oligonucleotides and permitting their joining through ligation.

If the conjugation is between free 3' ends these have to be designed to be mutually complementary to achieve base pairing and initiation of DNA synthesis extending the 3' ends of the the molecules.

Thus, only in those cases where the antibodies are brought close enough through binding to the same antigen molecule can the oligonucleotides be ligated. Ligated molecules subsequently serve as templates for nucleic acid amplification reactions.

In Fig. 2, there is shown a suitable way to attach the oligonucleotide extension to the antibodies. First, the oligonucleotides are terminally amino-modified and then attached to primary amines on the antibodies via disulphide bonds, e.g. according to the technique of Chue and Orgel, Nucleic Acid Research, Vol. 16, No. 9, 1988. Another way is by direct covalent coupling as described by Hendrickson et al., supra.

The antibodies used in the invention can be polyclonal, monoclonal or single chain antibodies produced by bacteriophages. In the latter case, it is possible to have antibodies equipped with an oligonucleotide binding part, rendering the above coupling step between antibody and oligonucleotide unnecessary.

The amplification technique to obtain detectable products is, for example, PCR (Polymerase Chain Reaction), LCR (Ligase Chain Reaction), SDA (Strand Displacement Amplification) bacteriophage

WO 97/00446

PCT/SE96/00779

6

Q $\beta$  replication, and 3SR (Self-Sustained Synthetic Reaction), of which the latter three methods do not require temperature cycling.

The method for detecting amplified products can, for example, be direct incorporation of a label, such as radioisotopes, fluorochromes, and enzymes, into the amplified products with the use of label-conjugated primers or nucleotides. Preferably, the accumulation of amplified products is monitored via the fluorescence from intercalating dyes, such as propidium iodide, etidium bromide and SYBR<sup>TM</sup> green from Molecular Probes.

The invention is not restricted to detection of any special kind of macromolecule, such as an antigen; the only criterion it has to fulfil is that it must be able to simultaneously bind three antibodies/affinity reagents. In the case where the affinity reagents are antibodies, the three antibodies are specific for different epitopes on the antigen. By biosensor analysis, it is possible to assure that the antibodies do not bind to overlapping epitopes on the antigen.

Examples of macromolecules are human myoglobin and human growth hormone. Ultrasensitive assays for growth hormone will have significant value in clinical situations where hormone levels are undetectable by prior art assays.

The invention will now be described below in a non-limiting Example.

#### EXAMPLE

Immunoglobulins were modified in a reaction with SPDP (3-((-pyridyldithio)propionic acid N-hydroxysuccinimide ester, from Pharmacia Biotech) according to the manufacturer's suggestions. Oligonucleotides were thiolated, either through the addition of a suitable phosphoramidite according to Connolly (Connolly BA, Nucl. Acid. Res. 1987 15:3131), or 3'aminomodified oligonucleo-



WO 97/00446

PCT/SE96/00779

7

tides were reacted with SPDP, followed by reduction of the dithiopyridyl bond, using dithiothreitol.

SPDP-modified antibodies were incubated with three equivalents of SH-containing oligonucleotides at 4°C over night. The reaction mixture was separated using a Zorbax HPLC gel filtration column. Residual free antibody were removed from the isolated conjugate by ion exchange MonoQ FPLC separation.

The two oligonucleotides used to conjugate the antibodies were Oligo 1: 5'Tr S C3-ATA GAC TGA GCG TGG ACA TTA ATA TGT ACG TAC GCT TAA TTG AGT 3' and Oligo 2: 5'P ATG TAC GAC CCG TAG ATA TTA TCA TAC TGG CAT GGG CAT GAT GAA CAT C-NHSPDP T3'

The immune test was performed by first binding 1µg of biotinylated antibody (#1) to individual streptavidin-coated prongs on a manifold support. [Parik et al., Anal. Biochem; (1993) 211: 144-150B]. After washes using PBS (phosphate buffered saline) with 0.5% Tween 20, the prongs were lowered into solutions of antigen (myoglobin) at variable concentrations. After further washes, the supports with bound antigen were incubated in a solution of two oligonucleotide-conjugated antibodies #2 and #3 at 5 ng each per reaction. The supports were washed, an oligonucleotide complementary to the free ends of the antibody-conjugated oligonucleotides was added (4 pmol per reaction, 5'CTA CGG GTC GTA CAT ACT CAA TTA AGC GTA 3'), and the ends of oligonucleotides on nearby antibodies were joined covalently by ligation at 37°C for 30 min using 1 U of T4 DNA ligase. The supports were then washed in a standard PCR buffer, and the supports were added as templates in a PCR mix, including two primers specific for sequences located at either side of the ligation junction (5'TTA ATG GCG AG 3') and Taq polymerase. After two cycles, the supports were removed and the amplification was continued for 26 more cycles. Amplification products were examined by separation in an agarose gel and ethidium bromide staining.

WO 97/00446

PCT/SE96/00779

8

## CLAIMS

1. An immunological test kit comprising a first immobilized reagent having affinity to a specific macromolecule, characterized in a second and a third affinity reagent specific for different determinantes of said macromolecule, and modified with crosslinkable oligonucleotides.
2. An immunological test kit according to claim 1, characterized in that the affinity reagents are antibodies, and that the macromolecule is a specific antigen.
3. An immunological test kit according to claim 1, characterized in that the affinity reagents are lectins, receptors, single chain antibodies, cofactors and nucleic acids.
4. An immunological test kit according to any one of the claims 1-3, characterized in that the oligonucleotides are complementary to each other.
5. An immunological test kit according to any of the claims 1-4, characterized in that it further comprises a ligase.
6. An immunoassay for detection of a specific antigen, characterized in:
  - a) contacting a sample suspected of containing said specific antigen with a first antibody linked to a solid support, said first antibody being specific for a first epitope on the antigen,
  - b) washing off excess reagents,
  - c) incubating with a solution of a second and a third antibody specific for a second and third epitope of said antigen, and modified with crosslinkable oligonucleotides enabling conjugation of said second and third antibody when both are bound to the said, same antigen,
  - d) washing off excess reagents,
  - e) amplifying said crosslinked oligonucleotides, and
  - f) detecting the amplified products.

WO 97/00446

PCT/SE96/00779

9

7. An immunoassay according to claim 6, characterized in that a ligase is added before step d).

8. An immunoassay according to claim 6 or 7, characterized in that an oligonucleotide complementary to the crosslinkable oligonucleotides is added before step d).

WO 97/00446

PCT/SE96/00779

1/1

FIG. 1

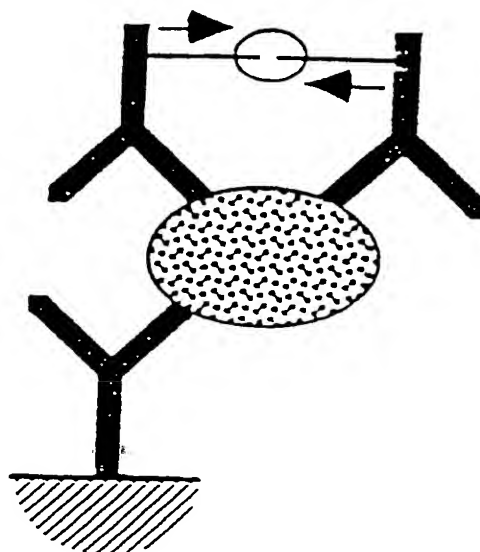
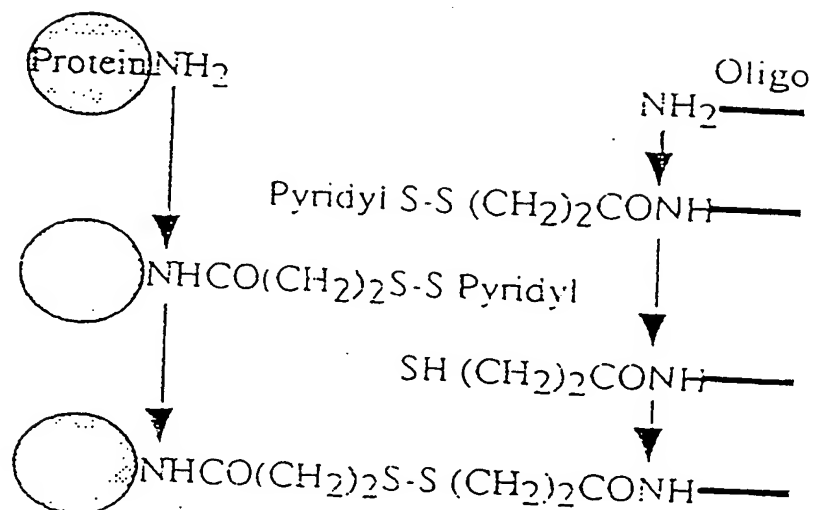


FIG. 2



SUBSTITUTE SHEET

1

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 96/00779

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
<b>IPC6: G01N 33/543 // C12Q 1/68</b> According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols)		
<b>IPC6: G01N</b>		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
<b>SE,DK,FI,NO classes as above</b>		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
<b>WPI, MEDLINE</b>		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMTECH, Volume 24, January 1995, Takeshi Sano et al, "Detecting minute amounts of antigen"	1-8
	--	
A	US 5219734 A (GARFIELD P. ROYER ET AL), 15 June 1993 (15.06.93)	1-8
	-- -----	
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
17 Sept 1996		18 -09- 1996
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86		Authorized officer  Carl-Olof Gustafsson Telephone No. +46 8 782 25 00

### Information on patent family members

International application No.

**PCT/SE 96/00779**

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
US-A-	5219734	15/06/93	AT-T-	120496	15/04/95
			AU-A-	2848589	27/07/89
			DE-D,T-	68921901	24/08/95
			EP-A,B-	0324616	19/07/89
			JP-A-	2005898	10/01/90
			US-A-	5449602	12/09/95
-----					

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**